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Complementary Combining Site Contact Residue Mutations of the Anti-digoxin Fab 26–10 Permit High Affinity Wild-type Binding*

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Antibody 26–10, obtained in a secondary immune response, binds digoxin with high affinity (K_a = 1.3 × 10^{10} M^{-1}) because of extensive shape complementarity. We demonstrated previously that mutations of the hapten contact residue HTrp-100 to Arg (where H refers to the heavy chain) resulted in increased specificity for digoxin analogs substituted at the cardenolide 16 position. However, mutagenesis of H:CDR1 did not result in such a specificity change despite the proximity of the H:CDR1 hapten contact residue Asn-35 to the cardenolide 16 position. Here we constructed a bacteriophage-displayed library containing randomized mutations at H chain residues 30–35 in a 26–10 mutant containing Arg-100 (26–10-RRALD). Phage were selected by panning against digoxin, gitoxin (16-OH), and 16-acetylgitoxin coupled to bovine serum albumin. Clones that retained wild-type Asn at position 35 showed preferred binding to gitoxin, like the 26–10-RRALD parent. In contrast, clones containing Val-35 selected mainly on digoxin-bovine serum albumin demonstrated a shift back to wild-type specificity. Several clones containing Val-35 bound digoxin with increased affinity, approaching that of the wild type in a few instances, in contrast to the mutation Val-35 in the wild-type 26–10 background, which reduces affinity for digoxin 90-fold. It has therefore proven possible to reorder the 26–10 binding site by mutations including two major contact residues on opposite sides of the site and yet to retain high affinity for binding for digoxin. Thus, even among antibodies that have undergone affinity maturation *in vivo*, different structural solutions to high affinity binding may be revealed.

The murine monoclonal antibody (Ab)[1] 26–10 (1, 2) binds digoxin (digoxigenin tridigitoxoside) (Fig. 1) with high affinity (1.3 × 10^{10} M^{-1}) exclusively through hydrophobic interactions arising from close shape complementarity (3). The specificity of Ab 26–10 for 33 structurally defined congeners of digoxin that vary in cardenolide ring substitutions, lactone ring saturation, and the number and nature of attached sugars has been characterized (4). Previously, we also explored the degree to which the interaction between this relatively rigid binding site and the rigid cardenolide structure could be altered *in vitro* to change affinity and specificity (5–7).

Ab 26–10 binds analogs substituted at position 16 of the cardenolide (Fig. 1) (4) less well than digoxin. The relative decrease in binding is proportional to the bulk of the 16-substituent (-OH, gitoxin; -CHO, 16-formylgitoxin; $-COCH_3$, 16-acetylgitoxin). The crystallographically determined structure of the complex between Fab 26–10 and digoxin (3) shows that the C16 position of the cardenolide contacts HAsn-35[2] in the first complementarity-determining region (H:CDR1) of 26–10 (Fig. 2). We therefore first constructed a bacteriophage-displayed library containing randomized mutations in H:CDR1 and panned this library against digoxin-BSA as well as the three 16-substituted analogs. Significant changes in specificity were not found. Virtually all mutants retained HAsn-35, which contacts hapten in the wt structure and hydrogen bonds with two other combining site residues (3).

Quite different results were obtained, however, when residues 99–101 (Kabat numbering (8)) of H:CDR3, located on the opposite side of the binding pocket to H:CDR1, were randomized (Fig. 2). When panned on 16-substituted congeners linked to BSA, a set of clones was selected with improved binding for the 16-substituted congeners with a concomitant decrease in affinity for digoxin. Sequence analyses of multiple mutants indicated that the mutation of tryptophan 100 to arginine was responsible for the specificity change, a result confirmed by site-directed mutagenesis (6). This result was unexpected as HTrp-100 lay on the side of digoxigenin opposite to the cardenolide C16 position (Fig. 2). HTrp-100 accounts for extensive contact with the cardenolide B, C, and D rings. We proposed that substitution of the wt Trp by Arg allowed the hapten to shift toward H:CDR3, thereby providing space for C16 substituents in the region of H:CDR1. In the present study, we planned to further explore possibilities for specificity modulation by looking for complementary or cooperative mutations on opposite sides of the binding site. An H:CDR3 mutant of 26–10, designated 26–10 RRALD (6), with an affinity of 1.9 × 10^9 M^{-1} for digoxin demonstrated a shift in specificity toward C16 analogs of digoxin. This mutant was used as a template for randomization at H:CDR1 positions 30–35 inclusive. RRALD represents a mutant sequence at positions 99–101 (Kabat numbering (8)). The corresponding wt 26–10 sequence is KWAMD. The phage-displayed library was panned against

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[1] The abbreviations used are: Ab, antibody; mAb, monoclonal antibody; Fab, antigen-binding fragment of antibody; V, variable region; CDR, complementarity-determining region; BSA, bovine serum albumin; ELISA, enzyme-linked immunoabsorbent assay; wt, wild type.

[2] H and L represent the heavy and light chain, respectively.

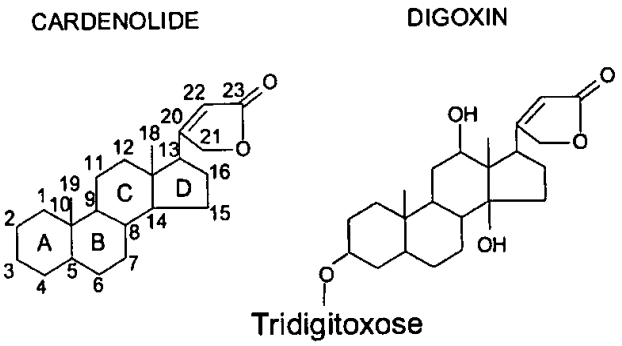

FIG. 1. **Schematic representation of the digoxin (digoxigenin tridigitoxoside) numbering system and steroid ring nomenclature.** The digoxin analogs used in panning are substituted at position 16 in the D ring (16-H, digoxin; 16-OH, gitoxin; 16-OCOCH3, 16-acetylgitoxin).

digoxin, gitoxin, and 16-acetylgitoxin, each coupled to BSA. Analysis of the sequences, binding specificity, and affinity of selected clones indicated that the mutation H:N35V[3] in H:CDR1 resulted in shifting the specificity back to wt. An unexpected observation was that certain Val-35-containing mutants also restored affinity for digoxin in the range of the wt 26–10. Thus, it proved possible to engineer *in vitro* an antibody with wt-like specificity and high affinity that contained mutations at two major contact residues, each of which when introduced independently lowered affinity for digoxin, thus revealing unexpected plasticity of the 26–10 combining site. This observation is in contradistinction to antibodies obtained following affinity maturation *in vivo*, where the central and contact residues of the combining site are generally conserved and little possibility of further structural differentiation was expected.

EXPERIMENTAL PROCEDURES

Vector Construction and Randomization of H:CDR1—Nucleotide sequences encoding the 26–10 Fab, from H chain position Glu-H1 to Arg-H228 (Kabat numbering (8)) in the hinge region (4, 9) and the entire L chain (Asp-L1 to Cys-L214), were introduced into the pComb3 vector (10) as described (6). 26–10 Fab was detected on the surface of bacteriophage M13 as a fusion protein with M13 protein 3. Excision of the gene 3 portion resulted in secretion of soluble 26–10 Fab into the culture supernatant (5) as described (10). Vector pComb3–26-10-RRALD was selected from a phage-displayed 26–10 library randomized at amino acids 99–101 in H:CDR3 (6). This clone produced Fab with lower affinity for digoxin ($K_a = 1.5 \times 10^9$ M^{-1}) relative to 26–10 wt but increased specificity for 16-substituted congeners. We used this as a template to introduce a cassette containing randomized residues at positions H30–35 inclusive (H:CDR1). DNA from the randomized H:CDR1 library (5) was cut with *Acc*I and *Aat*II, and DNA from the pComb3–26-10 RRALD vector was cut with the same enzymes. The 1413-bp RRALD fragment was gel-purified and ligated as described previously (5).

The resulting H:CDR1/RRALD library was introduced by electroporation into *Escherichia coli* XL1-Blue F′ cells. Infection of the XL1-Blue library with VCSM13 helper phage (XL1-Blue F′ and VCSM13 are from Stratagene, La Jolla, CA) generated a library of phage with surface Fab containing six substituted positions in H:CDR1.

Phage were recovered and concentrated by polyethylene glycol/NaCl precipitation from bacterial supernatants (10). Bacteriophage yield was quantitated by counting of ampicillin-resistant *E. coli* colonies on agar plates.

Site-directed Mutagenesis—The site-directed mutant 26–10 N35V was constructed as follows: an oligonucleotide complementary to positions 382–426 of the pComb3 vector, containing an *Mfe*I restriction site, and a reverse primer complementary to positions 489–512, containing the mutation N35V, were used to make a PCR fragment containing the sequence encoding Val-35 at the 3′ end. This PCR fragment was then used as a forward primer to obtain a larger PCR fragment with a *Bam*HI sequence at its 3′ end. The reverse primer was complementary to positions 876–897 and contained the *Bam*HI sequence. This 515-bp PCR fragment was cut with *Mfe*I and *Bam*HI, and the resultant 480-bp *Mfe*I/*Bam*HI fragment was cloned into the pComb3–26-10-RRALD vector. The double mutant 26–10 H:N35V/W100R was made by ligating two DNA fragments: a 2611-bp *Acc*I/*Aat*II fragment of the vector pComb3–26-10-RRALD containing the mutation H:N35V described above, ligated with a 1413-bp *Acc*I/*Aat*II fragment of pComb3–26-10-W100R. The construction of the pComb3–26-10-W100R mutant was described previously (6). The presence of the two mutations N35V and W100R in the final construct was confirmed by DNA sequencing.

Biopanning—Enrichment of the bacteriophage expressing specifically bound Fab mutants was achieved by successive rounds of binding to cardiac glycoside-BSA-coated microtiter wells (Costar 3690, Cambridge, MA) followed by washing, elution, re-infection, and growth according to the procedure of Barbas and Lerner (10). Cardiac glycoside-BSA coupling was described previously (5). Phage from each panning round was grown in *E. coli* without the VCSM13 helper, and phagemid DNA was isolated from a 50-ml culture using a Plasmid Midi Kit (Qiagen, Chatsworth, CA). An aliquot was sequenced to estimate the minimum complexity of the sequences in the mutated H:CDR1 region. Phage were analyzed after four successive rounds of biopanning. A 1-μg sample of pooled phagemid DNA from the final panning round was cut with *Nhe*I and *Spe*I and re-ligated to remove gene 3 (10). After re-ligation, the DNA was transformed into XL1-Blue cells and plated on Luria-Bertani medium/carbenicillin plates. Bacterial colonies were isolated and screened for Fab production and antigen binding by ELISA. The DNA sequences of cardiac glycoside ELISA-positive clones were determined using the dideoxy sequencing method. Fab was produced as described previously (5).

ELISA—Bacterial supernatants were tested in a direct binding ELISA in 96-well microtiter plates coated with BSA alone, cardiac glycoside-BSA, or goat anti-mouse Fab antibody (ICN, Costa Mesa, CA) (5). Briefly, wells were coated with antigen and blocked with 3% BSA in 0.1 M sodium phosphate buffer, 0.15 M NaCl, 0.02% sodium azide. Bacterial supernatant was added and incubated for 3 h at room temperature and detected after a 2-h room temperature incubation with peroxidase-labeled F(ab′)$_2$ fragment of goat anti-mouse IgG and IgM using the TMB Microwell Peroxidase Substrate System (Kirkegaard and Perry Laboratories, Gaithersburg, MD) and neutralization with 1.0 M phosphoric acid. Color development was measured at 450 nM in a Bio-Tek ELISA reader.

Affinity and Specificity Determination—Affinities for digoxin were measured on dilute bacterial supernatants using a saturation equilibrium assay (5) by using filtration through glass fiber filters to separate bound and free [^{3}H]digoxin (New England Nuclear, Boston, MA). Each tube contained a constant amount of Fab, one of twelve serial dilutions of [^{3}H]digoxin (1.1×10^{-7} M to 3.0×10^{-11} M), depending on preliminary screening, and 0.5 μg/ml goat anti-mouse Fab antibody. The goat anti-mouse Fab antibody was necessary to efficiently retain the anti-digoxin Fab on the filter (5). Scatchard analysis was used to determine affinities.

The specificity (relative affinity) of Fab for different cardiac glycosides was determined using a competition ELISA assay, described previously (7). Wells of microtiter plates were coated with either digoxin-BSA, gitoxin-BSA, or 16-acetylgitoxin-BSA and contained a constant amount of Fab and serial dilutions of cardiac glycosides. Each mutant Fab was tested on ELISA with the 16-substituted congeners and digoxin as competitors. The values reported (see Table I) are ratios of the molar concentrations of inhibitor required to give 50% inhibition of the binding of Fab to cardiac glycoside-conjugated BSA-coated wells relative to (divided by) the molar concentration of digoxin that gave 50% inhibition.

RESULTS

Previously, by panning from a randomized 5-mer H:CDR3 library (positions 99–101) displayed on bacteriophage, we selected mutants of the anti-digoxin Fab 26–10, which preferentially bound to analogs of digoxin substituted at the C16 position of the cardenolide (6). The shift in specificity proved to be attributable to the substitution of Arg for Trp at position 100. The mutant 26–10-RRALD (residues 99–101) was chosen as a parent clone for construction of a randomized H:CDR1 library (residues 30–35 inclusive) (5) to examine the effects on affinity and specificity in the presence of mutations on the opposite side

[3] Mutants are denoted by the position number, preceded by the wt or parental residue and followed by the mutated residue.

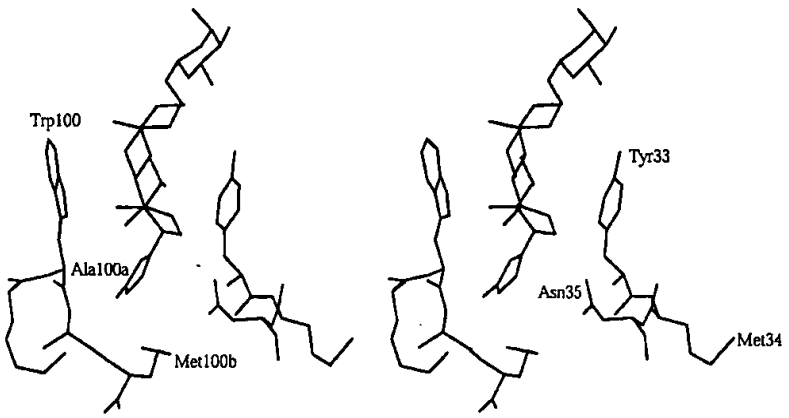

FIG. 2. **Stereo view of digoxin (digoxigenin tridigitoxoside) with portions of H:CDR1 and H:CDR3 from the x-ray crystal structure of the 26–10 Fab:digoxin complex (3).** Digoxin is at the *center* with the lactone at the *bottom*. At the *top* is shown a single attached sugar (digoxigenin monodigitoxoside); H:CDR3 (positions 99–101) is on the *left*; the residues that contact digoxin are *labeled* explicitly (Trp-100, Ala-100a, Met-100b). H:CDR1 is on the *right*; Tyr-33 and Asn-35 contact the hapten.

of the combining site.

The original H:CDR1 library (5) contained 10^7 clones and represents the maximum diversity in the H:CDR1-RRALD library constructed here. (Because this is a second generation library made from the first Maxi-Prep of the H:CDR1 library and an insert containing H:CDR3 RRALD, it is not possible to determine the library size by counting colonies.) The library was panned four times on digoxin-BSA, and two congeners of digoxin substituted at C16: gitoxin (16-OH) and 16-acetylgitoxin coupled to BSA. Secreted Fab were tested for binding to digoxin-BSA and goat anti-mouse Fab antibody. More than 90% of clones selected after four rounds of panning for each cardiac glycoside bound digoxin in an ELISA. Twenty-five digoxin-binding clones were selected from pannings on each of the three cardiac glycosides. DNA sequence analyses of 21 clones from panning on digoxin-BSA, 20 from gitoxin-BSA, and 22 from 16-acetylgitoxin-BSA are shown in Table I. Several sequences were observed more than once. Sixteen unique sequences were found following panning on digoxin-BSA, 18 for gitoxin-BSA, and 17 for 16-acetylgitoxin-BSA.

The affinities (K_a) for digoxin were measured for most clones (Table I), revealing a range of ≤ 0.2 to 10.5×10^9 M^{-1}. The average affinity[4] for clones selected using digoxin-BSA was 4.5×10^9 M^{-1} (including duplicates), 1.06×10^9 M^{-1} for gitoxin-BSA-selected clones, and 1.54×10^9 M^{-1} for 16-acetylgitoxin-BSA-selected clones. By comparison, the affinity of wt 26–10 in parallel assays was 13×10^9 M^{-1}, and the affinity of the parental mutant 26–10-RRALD was 1.9×10^9 M^{-1}.

Specificities of mutant Fabs for the three analogs were determined using competition ELISA for representative clones. In general, two different specificity patterns were found in all groups selected by all three analogs: one similar to the parent RRALD mutant (increased relative affinity for 16-substituted analogs) and the other resembling that of the wt 26–10 specificity, where digoxin is bound to a greater extent than 16-substituted analogs. There was an absolute correlation between the presence of a Val residue at position 35 and wt-like specificity (Table I); in addition, Asn at position 35 was always associated with heteroclitic binding to gitoxin, similar to the parent mutant 26–10-RRALD.

Among 16 clones, each with a unique sequence selected on digoxin-BSA, all but one expressed Val at position 35. As noted above, digoxin-BSA-selected clones exhibited significantly higher affinity for digoxin and wt-like specificity associated with the presence of HVal-35. Among 18 gitoxin-BSA-selected clones, eight expressed valine, and for the larger analog 16-acetylgitoxin, one of 17 clones expressed Val at position 35. Asn-35, the wt 26–10 residue, was not observed among digoxin-BSA-selected clones but was present frequently in clones selected by the other congeners, as was Ser-35.

To test the effect of the mutation N35V independently of other H:CDR1 residue mutations, we constructed by site-directed mutagenesis the mutant 26–10-RRALD-N35V (Table I). This single mutation resulted in a 3-fold increase in affinity for digoxin relative to 26–10-RRALD, associated with reversion to the wt-like specificity (Table I). We also constructed a double mutant, H:N35V/W100R, on the wt background. The affinity of this mutant Fab for digoxin was 3-fold lower than that of 26–10-RRALD. The affinity of the mutant 26–10-KRAMD constructed previously (6) and that of 26–10-RRALD are approximately the same. Thus, the mutation N35V has opposite effects upon affinity when combined with the sequences KRAMD *versus* RRALD at positions 99–101. Accordingly, the effects on the affinity of double mutations at the contact residues at positions 35/100 depend on the context of other mutations in H:CDR3. Residues other than H35 in CDR1 also modulate affinity as only certain RRALD mutants with Val-35 have improved affinity (Table I). However, the specificity of the 26–10 H:N35V/W100R mutant is reverted to wt-like specificity as in *all* selected clones (Table I) and other site-directed mutants, indicating a consistent correlation of specificity with the identity of the residue at H35.

Amino acid substitutions at positions H30–32 were diverse (Table I). At position 33, where Tyr is a hapten contact residue in the crystal structure of the Fab 26–10:digoxin complex (3), an aromatic residue was present in 35 of 51 clones. At position 34, which points into the hydrophobic core of V_H, only the aliphatic residues Ile, Leu, Val, and the aromatic Tyr and Phe were observed. The distribution of amino acid substitutions at residues 30–34 in the experiment reported here using a 26–10-RRALD parent was very similar to that determined previously in experiments using the wt 26–10 as the parental clone for H:CDR1 mutagenesis (5). The conservation of these residues is in accordance with the hypothesis that the general binding site motif is maintained in the mutants expressing non-wt residues at the nominal contact residues H35 and H100. However, in the wt background, HAsn-35 is nearly always selected (5), in contrast to HVal-35 when the parental clone is 26–10-RRALD.

[4] The lower limits of measurement in the affinity assay are $\leq 5 \times 10^6$ M^{-1} (4). The calculations of average affinity assume, for these digoxin-binding clones, $K_a = 0.01 \times 10^9$ M^{-1}.

TABLE I

Amino acid sequences, affinity for digoxin, and specificity of binding to digoxin congeners of clones selected by digoxin-, gitoxin-, or 16-acetylgitoxin-BSA from a 26–10-RRALD Fab phage library randomized at H chain CDR1 positions 30–35

Fab[a]	Affinity[b] × 10^9 M^{-1}	H chain amino acid sequence positions 30–35	Ratio of inhibitory concentrations[c]		
			D	G	A
wt 26–10	13.0	TDFYMN	1	3	100
26–10-RRALD	1.9	TDFYMN	1	0.4	54
26–10-RRALD-N35V[d]	5.5	TDFYMV	1	10	210
D1	1.9	RKSYVV			
D2[e]	6.5	FNFYYV	1	11	48
D3[e]	6.2	PHAYIV	1	6	64
D4	ND[f]	SDYTIV			
D5	3.9	INYWLV	1	5	70
D6	0.3	NPGAIV			
D7	0.6	PQLTYV			
D8	2.0	KRHWLV	1	10	
D9	3.6	FSLYLV			
D10	1.1	GPGFVV			
D11	1.7	WTNYIV			
D12[e]	5.8	PNFYIV	1	10	61
D17[e]	10.5	EDLYIV	1	10	61
D18	NM[g]	KNLRIS			
D20	1.2	KRHWLV			
D22[e]	6.6	SDTFIV	1	9	83
G1[h]	1.2	RPAYFN	1	0.2	43
G2	0.4	PPLHVV			
G3	0.6	RGFVFS			
G4	3.0	EESFLV	1	8	48
G5	NM	RHSTVV			
G7	ND	NKGTVV			
G8	1.0	RDGYIS			
G9	NM	RYGWIT			
G10	8.2	YDTWYV	1	5	30
G11	0.3	PPKFLN			
G12	0.9	PSSWLN	1	0.5	65
G13	0.2	PSSHIV			
G14	NM	PYATIV			
G15	1.0	RRTEIS			
G16	0.2	GHDYIT			
G17	NM	PFLSIV			
G18	0.7	KGMWFS			
G19	0.3	PGHMIN			
A1	0.2	NRHFIA	1	11	30
A2	0.5	GPRYLS	1	2	56
A3[i]	NM	HDFTFG			
A6	0.4	RAPSIA	1	3	69
A7	1.2	TNYFFN	1	0.2	67
A8	1.5	NDWYIN	1	0.2	65
A9	1.5	PGNWLS	1	2	72
A10[e]	2.9	SDLYLA	1	21	37
A12	1.1	NNSWIN	1	0.1	17
A13	1.9	RHSYVN	1	0.3	50
A14	NM	GPRKIF			
A15	3.6	RDNFFV	1	6	55
A16	NM	NNATIG			
A17	1.2	RPAYIS	1	4	89
A22	7.1	PAYYLS	1	18	78
A23	2.2	MPTWLS			
A25	0.9	NGFCFS			

[a] Wild type (wt) 26–10 and the 26–10 mutant Fab RRALD containing the amino acids RRALD at heavy chain positions 99–101 are compared with Fab from 26–10 RRALD mutants randomized at H:CDR1 positions 30–35 selected by panning against digoxin-BSA (D), gitoxin-BSA (G), or 16-acetylgitoxin-BSA (A). Mutant clones are designated by a single initial (D, G, or A) for the glycoside used in selection followed by the sample number for each clone.

[b] Affinities were measured using an equilibrium saturation method with filtration through glass fiber filters for separation of bound and free ligand (5, 25). The margin of error between the determinations for a given Fab in different experiments was less than 25%.

[c] The ratio of inhibitory concentrations is the ratio of the molar concentrations of cardiac glycoside congener to the molar concentration of digoxin that inhibits 50% of binding of digoxin to Fab.

[d] 26–10 RRALD-35V was constructed by site-directed mutagenesis using 26–10 RRALD as template.

[e] Identical sequences found twice.

[f] ND, not determined.

[g] NM, not measurable in this assay ($K_a \leq 5 \times 10^6$ M^{-1}).

[h] Identical sequence found three times.

[i] Identical sequence found five times.

DISCUSSION

The 26–10 mutant with sequence RRALD at positions 99–101 in H:CDR3, obtained previously by selection from a phage-displayed library (6), has an affinity for digoxin of 1.9×10^9 M^{-1} as compared with the wt affinity of 13×10^9 M^{-1}. The wt sequence in this region is KWAMD. Extensive consensus sequences indicated that the mutation H:W100R was responsible for improvement in specificity for 16-conjugated digoxin ana-

logs associated with a decrease in affinity for digoxin. A site-directed mutant, 26–10-W100R, resulted in a 6- to 7-fold decrease in affinity for digoxin (6), associated with increase in relative binding to C16-substituted digoxin analogs. In the work reported here, substitution of Val-35 for the wt Asn in H:CDR1 on the background of 26–10 RRALD restored digoxin binding and 26–10-like specificity in several clones (Table I), apparently also dependent on the context of other residues in H:CDR1. Among 14 clones with affinities for digoxin higher than that for the parent clone 26–10-RRALD, eight were selected by digoxin. All retained the wt HTyr-33 or the aromatic substitutions Trp or Phe. HTyr-33 is a contact residue to digoxin in the wt 26–10 Fab:digoxin complex (Fig. 2). The preponderance of Tyr at H33, as well as the strong preponderance of aliphatic Leu, Val, Ile, and aromatic residues at position 34, which points into the hydrophobic core of V_H in the wt structure, suggest that the local conformation of H:CDR1 is conserved to a considerable degree in the mutant clones containing Arg-100 and substitutions for wt HAsn-35, particularly Val. A similar set of consensus amino acids was observed (5) at positions 33 and 34 in randomized H:CDR1 libraries using, as template, the wt 26–10 in which the wt residue at position 100 is Trp. We also showed previously that wide variations in amino acid side chains were permissible at residues 30–32 of the H:CDR1 loop in the wt 26–10.

To test the proposal that the substitution of Val at position 35 in 26–10 RRALD is responsible for increasing digoxin binding and reversion to wt specificity, we constructed the mutant 26–10 RRALD N35V. This mutant demonstrated a 3-fold increase in digoxin binding and a reversion to wt specificity (Table I). However, the same mutation (N35V) in the wt background reduces digoxin binding more than 90-fold (11). This last result is consistent with previous analyses of digoxin-BSA-selected mutants from an H:CDR1 library of wt 26–10 (5), where virtually all digoxin-binding clones retain the wt Asn at H35 and specificity shifts were not observed.

Thus, combined non-conservative mutations at two contact residues, H35 and H100, each of which separately results in a significant decrease in affinity for digoxin, taken together restore digoxin affinity in some instances (*e.g.* clone D17) nearly to wt 26–10 levels. This result is particularly unexpected as each of the two residues in the wt accounts for significant interactions with hapten. HTrp-100 makes extensive van der Waals contact with residues from the B, C, and D rings of digoxin. On the other side of the binding pocket, there is tight complementarity between HAsn-35 and the C16 position of the cardenolide (3). HAsn-35 forms hydrogen bonds with the hydroxyls of HSer-95 and HTyr-47, two other hapten contact residues. Mutagenesis limited to position H35 resulted in decreased affinity for digoxin (11). Bulkier substituents ablated binding, consistent with tight complementarity between HAsn-35 and the cardenolide. HAsn-35 is involved in hydrogen bonding to other residues in a majority of Fab crystal structures (summarized by Padlan (12)) and is thought to thereby stabilize the binding site in many antibodies. Although this may be the case in the wt 26–10, where the H:N35V substitution is deleterious, in the reordered combining site of the 26–10 mutants described here, containing Val-35 and Arg-100, this hydrogen bond network is not critical to effect high affinity digoxin binding. We had assumed that these bonds resulted in partial compensation to HSer-95 and HTyr-47 for the lack of solvent and the general hydrophobic environment when digoxin is bound. The preponderance of Val at H35 among selected mutants and the increased affinity of the mutant N35V would argue, however, that the absence of these hydrogen bonds is not as deleterious as expected (11).

We reported previously cooperativity between two hapten contact residues to increase the affinity for a cardiac glycoside analog (7) for Fab 26–10 in which a 26–10 phage-displayed mutant containing the mutation W100R was further mutagenized in the H:CDR3 region at positions 94–98 and mutant phage selected on 16-substituted digoxin analogs. A set of mutants was recruited with further increase in specificity for 16-substituted haptens due to the additional contact residue mutation H:S95G. These mutants bound gitoxin (16-OH) with affinities up to 150-fold greater than that of the wt 26–10 for digoxin. Cooperativity between contact residues was also reported previously for the germline-encoded Ab McPC603 (13).

The finding that two contact residue mutations in a high affinity Ab obtained in a secondary immune response provide an alternative mode for high affinity hapten binding has not, to our knowledge, been observed during affinity maturation *in vivo*, where in general mutations that enhance affinity occur in non-contact amino acids or even amino acids distant from the site (14–21). Mutagenesis studies of contact residues of Abs where the structure has been determined crystallographically indicate that mutations of contact residues most often result in a significant decrease in affinity for antigen (although a few contact residues may be energetically unimportant) (11, 13, 14, 17, 22, 23). Inasmuch as affinity maturation *in vivo* involves stepwise selection and expansion of mutant B cell clones with resultant enhanced affinity, mutations such as 26–10-H:W100R or 26–10-H:N35V would be unlikely to be selected *in vivo*, although there are rare examples of recruitment of low affinity clones (24). Engineering of Abs *in vitro*, however, allows sets of cooperative mutations to be revealed irrespective of the individual contributions of each residue and independent of the temporal order of mutations.

For 26–10, comparison of the unliganded Fab and the ligand-bound Fab demonstrates superimposition of structure without evidence for induced fit. Nonetheless, despite our previous assumption that 26–10 would have limited plasticity following differentiation during the secondary immune response, resulting in high affinity, it is possible to produce mutants *in vitro* with alternative contact residues that satisfy the requirements of high affinity binding to digoxin. Whether this unexpected diversity is related to a binding site mode dependent on pure hydrophobic interactions, with exclusion of water, or whether it may occur in other binding sites involving additional types of interactions or those differing in shape is not known.

Affinity maturation *in vivo* proceeds via a selection mechanism that strongly favors single-point somatic mutations that are either neutral or produce enhanced antigen affinity, whereas mutations that reduce affinity are disfavored. If this is the case, then the selection of two mutations that individually reduce affinity but together show increased affinity may be a rare event *in vivo*. The final end product of antibody maturation may be as much a product of the *path* of mutation from germline to secondary antibody as a true optimization of antibody-antigen interactions.

In the course of antigen-driven selection *in vivo*, alternative contact residues resulting in higher affinity binding have not been observed. The findings reported here, in which mutations of two contact residues distant from each other result in the formation of a binding site that is functionally equivalent to the wt, indicate that even high affinity secondary-response Abs may exhibit greater structural combining site differentiative capacity during the course of mutagenesis *ex vivo* than expected.

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